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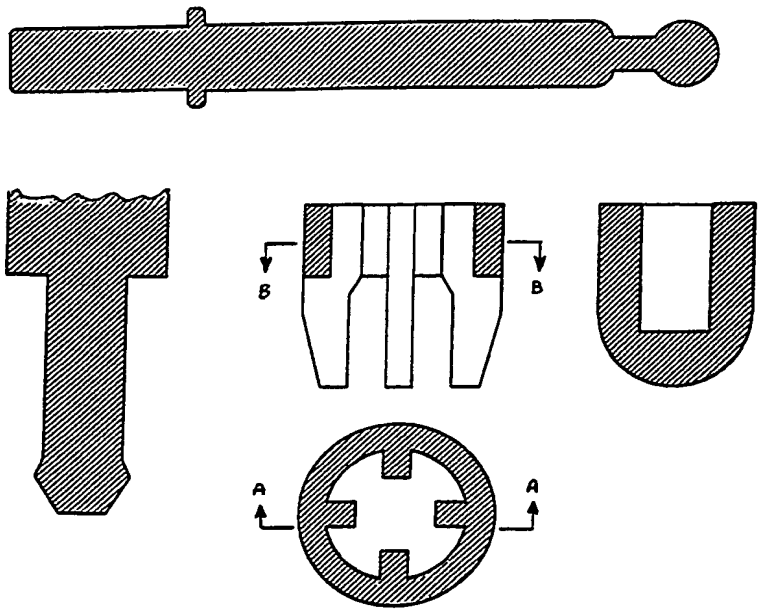
(54) Title: SOLID SURFACE FOR PEPTIDE SYNTHESIS



(57) Abstract

There is disclosed a solid surface, such as the surface of a rod or pin, for use in the synthesis of polymeric compounds such as peptides. The surface comprises an active region and an inactive region being positioned such that, in use, it is below the surface of the reaction liquid in a reaction well, irrespective of minor variations in liquid levels between various wells so as to eliminate the formation of deletion compounds. There are further described such surfaces for the formation of relatively large quantities of compound wherein the active region has a higher surface area/volume ratio than that of a cylinder of corresponding volume.

* See back of page

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"SOLID SURFACE FOR PEPTIDE SYNTHESIS"

This invention relates to a solid surface for the synthesis of peptides thereon, and in particular relates to a rod or pin having a surface on which a peptide may be synthesized efficiently and economically.

In prior International Patent Specification No. PCT/AU84/00039, there is disclosed a method for the simultaneous synthesis of a large number of different peptides. Basically, this method involves the synthesis of peptides onto a solid polymeric surface, such as polyethylene, which is molded into the shape of a rod or pin. In a preferred embodiment of the method (but not limited to it), these rods or pins are positioned in a holder so that they form a 12 by 8 matrix, with the rods or pins being positioned so that the spacing corresponds to that of the wells of microtitre plates which are widely used for ELISA (enzyme-linked immunosorbent assay) tests.

The method disclosed in this prior specification was based on the realization that for the solid-phase synthesis of any peptide, almost all of the steps of the synthesis are exactly the same for any peptide and are independent of the sequence of the peptide being synthesized. Thus, with the rods or pins arranged in the preferred format so that 96 are in a holder, all steps in the synthesis such as washing steps, neutralization steps and deprotection steps can be carried out simultaneously in the synthesis of 96 different peptides. The only steps which must be carried out separately for each

different peptide are the coupling of the appropriate amino acid residues. Each of these steps is conveniently carried out by dispensing appropriate activated amino acid solution into the corresponding wells of a microtitre plate or the like. Thus, the appropriate amino acid is coupled to the peptide for each of the rods.

The quantity of peptide covalently bonded to the solid polymer surface by this method is sufficient to allow reaction of the peptide with specific binding entities such as antibodies to be readily detected. Although the quantity of peptide synthesized on each rod is relatively small (being of the order of tens of nanomoles), the ability to reuse the synthesized peptide after a test compensates for the small quantity of peptide on the rod. However, in some applications the quantity of peptide needs to be greater. Examples of such applications include the removal of the peptide from the rod and recovery of specific binding entities for further testing. Accordingly, modifications to the process of synthesis and testing of the peptides have been disclosed in Australian Patent Specification No. PJ2788/89.

It is an object of the present invention to provide means by which the amount of peptide, or for that matter any other polymeric compound such as nucleic acid which can be synthesized on a solid support, can be increased while retaining the advantage of being able to synthesize many peptides with different sequences simultaneously.

In the earlier method disclosed in International Patent Specification No. PCT/AU84/00039 polyacrylic acid was grafted to the surface of a solid polyethylene support using gamma-irradiation. In that earlier system, the region on the rod on which peptide was grafted was completely defined by the volume of reagent used for the

coupling of specific amino acids to the growing peptide, or more accurately, the depth into which the rod dipped into the amino acid solutions. As this depth inevitably varied slightly from cycle to cycle in the synthesis, the result was a small region on the rod where the peptide synthesized may have had appreciable amounts of deletion peptides (that is, peptides whose sequence varied from that intended by having one or more residues absent) present because slightly less of one of the activated amino acid solutions was dispensed in one or more cycles.

A further disadvantage of the earlier system is that the polymer layer grafted onto the rods during the radiation process is readily solvated by many solvents and as a consequence, solvents will migrate upwards through this layer by capillary action. This results in depletion of the reservoir of reagent and consequently, as described above, a larger zone of uncertain synthesis quality is created. In addition, unless extreme care is taken in extensively washing these rods, the polymer layer acts as a reservoir of the solvent used in synthesis leading to the contamination of subsequent solutions.

In a first embodiment of the present invention, there is provided a surface for use in the synthesis of peptides or other polymeric compounds thereon, which surface comprises an active region on which said synthesis may take place and an inactive region on which said synthesis will not take place.

Preferably, the surface is the surface of a rod or pin, more particularly a rod or pin of the general type disclosed in International Patent Specification No. PCT/AU84/00039, having a portion providing the active region and remaining portion providing the inactive region.

Preferably also, the portion of the rod or pin providing the active region of the surface on which the synthesis may take place is of a shape or construction which provides an increased ratio of surface area-to-volume in this active region.

By way of illustration, Figure 1 represents a cross-sectional drawing a rod or pin. The 4.0 mm diameter sphere at one end will, after appropriate treatment, become the active region. This can be conveniently done by allowing only this region of the polyethylene pin to contact the acrylic acid solution during gamma-irradiation as disclosed in International Patent Specification No. PCT/AU84/00039. Thus, only this region of the pin will have polyacrylic acid grafted to it and consequently, only on this region of the pin or rod will synthesis be possible. Thus, the amount of peptide synthesized will be independent of the volume of reagents used during synthesis so long as the depth of the solutions into which the rods or pins are immersed is sufficient to cover the active region during the coupling step. In this way, the possibility of deletion peptides will be reduced to a minimum. Furthermore, because the polyacrylic acid is grafted only on the active region, there is no possibility of solvent migration through capillary action as described above for the earlier system. Finally, because less polyacrylic acid has been grafted on to the surface of the rod or pin, the risk of contamination of subsequent solutions through "carry over" of solvents is correspondingly reduced.

In a second embodiment of the present invention there is provided a solid surface for use in the synthesis of peptides or other polymeric compounds thereon, which surface comprises an active region which is of a higher surface area/volume ratio than that of a cylinder of

corresponding volume.

In another embodiment of the invention, the active region may be provided by a surface coating of a suitable resin applied to it. The coating can be made of any of the porous resins which are used for conventional solid phase peptide synthesis. Because these resins are porous, the surface area of the active region is increased dramatically and so allows a much greater yield of peptide or other polymer to be synthesized. In addition, the use of this embodiment of the invention makes it particularly convenient to change the chemistry used in the synthesis, and indeed, change the class of polymeric compounds to be synthesized by selecting the appropriate resin with which to coat the rod or pin. Examples of porous resins which may be used in such coatings include benzhydrylamine-polystyrene resin and polyacrylamide gel inside kieselguhr.

In a particularly preferred embodiment of the invention, the portion of the rod or pin providing the active region of the surface is manufactured as a separate entity, and then combined or assembled with the remaining portion of the rod or pin. Preferably, in this embodiment, the separately manufactured portion is a "tip" portion of the rod or pin, with the remaining portion being a "shaft" portion. This approach has two major advantages:

Firstly, the materials from which the portion providing the active region is manufactured may be selected mainly for their ability to act as a suitable base for peptide synthesis and only minimal regard given to other qualities such as mechanical strength. For instance, a soft, flexible plastic polymer can be chosen for the active region and the need to rigidly maintain the active region in a particular format can be

disregarded. In another example, the portion providing the active region could be manufactured by sintering together spherical particles of glass, ceramic or other suitable materials. Furthermore, the remaining portion providing the inactive or support region may be constructed of a material selected solely for its mechanical properties and which may not be suitable at all for peptide synthesis. By way of example, this portion may be constructed from polypropylene or some other non-porous plastic material.

Secondly, the surface of the portion providing the active region will usually need to be treated to enable peptides or other polymers to be synthesized on to its surface. One suitable method disclosed in International Patent Specification No. PCT/AU84/00039 involves the grafting of polyacrylic acid onto the surface of a solid polyethylene support using gamma-irradiation. Dividing the rod into two functional portions - one providing the active region and another providing the inactive or support region also ensures that syntheses are more controlled. If the active region is the only component of the system which has been treated to allow synthesis of the peptide or other polymer, then synthesis will be restricted to it and it alone, in contrast to the earlier system, in which the complete rod was grafted with polyacrylic acid. In that earlier system, the region on the rod on which peptide was grafted was completely defined by the volume of reagent used for the coupling of specific amino acids to the growing peptide, or more accurately, the depth into which the rod dipped into the amino acid solutions. As this depth inevitably varied slightly from cycle to cycle in the synthesis, the result was a small region on the rod where the peptide synthesized may have had appreciable amounts of deletion peptides (that is, peptides whose sequence varied from that intended by having one or more residues absent)

present because slightly less of one of the activated amino acid solutions was dispensed in one or more cycles. If the portion providing the active region is manufactured as a separate entity as described above, and its surface treated then only the active region will permit coupling of the amino acids or other monomers. Thus, the amount of peptide synthesized will be independent of the volume of reagents used during synthesis so long as the active region is covered by solution during the coupling step.

Another major advantage of manufacturing the portion providing the active region as a separate entity is the minimization of cross-contamination of solutions. The polymer layer grafted onto the rods during the radiation process as described in International Patent Specification No. PCT/AU84/00039 is readily solvated by many solvents and as a consequence, solvents will migrate upwards through this layer by capillary action. This results in depletion of the reservoir of reagent and consequently, as described above, a larger zone of uncertain synthesis quality is created. Also, unless extreme care is taken in extensively washing these rods, the polymer layer acts as a reservoir of the solvents used in synthesis leading to the contamination of subsequent solutions. Where the portion providing the active region is manufactured separately as described herein, this migration of solvents and reagents cannot take place.

Another major advantage of manufacturing the active region as a separate entity comes about because the active region will typically be much smaller than the complete unit. Therefore, more of the active region components can be treated simultaneously to create the active region with consequent savings in materials and time.

After treatment, the separately manufactured portions providing active regions (for example, tip portions) may be attached to the remaining portions (for example, shaft portions) by any suitable means. These include, but are not restricted to, heat fusing the active tip portion to the shaft portion; using a suitable adhesive to glue the active tip portion to the shaft portion; the mechanical attachment of the active tip portion to the shaft portion.

As previously described, it is preferred that the portion providing the active region be of a suitable shape or construction to provide an increased surface area/volume ratio in the active region. Such as increased surface area/volume ratio increases the amount of peptide or other polymer which can be synthesized when compared with, for example, a solid cylindrically shaped portion.

For the purpose of illustration only, the portion providing the active region of the rod, that is, the region of the rod on which the peptide or other polymeric molecule is to be synthesized, is a cylinder with a radius of 2 mm and a height of 4 mm, the surface area of which active region is 61.8 mm^2 (assuming that only one end of this cylinder is available for synthesis). However, if a slit 1mm wide is made across the diameter of this portion of the rod, the surface area becomes 81.8 mm^2 (1.3 times the area of the solid cylindrical portion). The surface area of the portion providing the active region can be increased even further by modification of the shape of this portion of the rod. For instance, if eight slits, each 0.4 mm wide and 1mm deep are made into the surface of a cylindrical portion, the surface area available for synthesis is increased to 124 mm^2 (twice the area of the unmodified cylindrical

portion). It will be apparent that the surface area of the active region of the rod can be further increased by further modification of its geometric shape, and equally that such modifications can be made by molding the rod in its final desired shape or by machining a molded rod into its final desired shape.

However, in a preferred embodiment of the invention, the portion providing the active region of the rod is made by joining small particles of solid materials together, for instance, by sintering using pressure or heat or both. This can be particularly useful where it is desired to use particular harsh chemistries or virulent solvents. For instance, glass is resistant to most solvents which would make most conventional plastic materials unstable. Thus an active region could be made by sintering together small spherical beads of glass. This could then be treated, for instance, by functionalizing the surface with an amino-silane, to make it suitable as a base on which the peptide or other polymeric compound could be synthesized. In this example, the inactive region would be made from a particularly resistant plastic such as polytetrafluoroethylene. In this way, a material such as glass, which would be an unsuitable material for the inactive region, can be used with advantage in the active region.

A further advantage of this particular embodiment of the invention is the large increase in the surface area/volume ratio of the active region can be achieved. Using the example given above, 8885 spherical rigid particles each with a radius of 0.1 mm would occupy the volume of the portion providing the active region of the rod if close packed together. The surface area of these spheres would be 1116.4 mm^2, 17.8 times the surface area of a solid cylindrical portion. Decreasing the size of

the particles to be sintered together, would provide a corresponding increase in the surface area available for synthesis. For example, decreasing the radius of the rigid spheres to be sintered to 0.055 mm increases the surface area to 2233 mm^2, about 35.5 times that of the solid cylinder. In practice, because of the process of joining the particles together, and the fact that the particles are neither uniform in size nor rigid, the theoretical increase in surface area would not be achieved. However, very significant gains in surface area available for synthesis can be achieved by making the solid support by sintering small particles of material together.

Further features of the present invention are illustrated, by way of example only, in the accompanying drawings. In Figure 2 there are shown two possible modifications which may be made to the geometric shape of the portion of the rod or pin providing the active region in order to increase the surface area of this active region. It will be appreciated that many other similar alterations of the geometric shape may be made to achieve a similar result.

Figure 3 illustrates, by way of example only, the two-part construction of a rod or pin of the general type disclosed in International Patent Specification No. PCT/AU84/00039. In this Figure (a) shows in longitudinal section the region of the "shaft" portion of the rod or pin on which is to be assembled the separately manufactured and treated "tip" portion. As will be seen from (a), this region is constructed so as to enable the tip portion to be retained on the narrowed section. The region shown in (a) is, of course, the end of the shaft portion of the rod or pin which in use will be remote from the holder in which the rods or pins are positioned. In Figure 3, (b) and (c) show two alternative

configurations for the "tip" portion of the rod or pin which may be separately manufactured and treated to provide the active region. In (b), there are shown both the longitudinal section (along the line A-A) and cross section (along the line B-B) of one configuration which provides a "vaned" tip. In (c), there is shown in longitudinal section a more simple tip configuration which may, for example, be either a solid plastics tip or a molded tip made up of sintered small particles.

Further features of the present invention are illustrated, by way of example only, in the following example.

EXAMPLE 1

10000 of the "vaned" tips illustrated in Figure 3(b) (with an external diameter of 5 mm) were gamma-irradiated in 1 litre of 6% acrylic acid solution as described in International Patent Specification No. PCT/AU84/00039. The tips then had hexamethylenediamine (HMD) coupled to the grafted polyacrylic acid by exposing them to a solution of 36.48 g t-butoxycarbonyl-hexamethylenediamine HCl, 24 ml triethylamine (TEA), 30.95 g dicyclohexylcarbodiimide (DCC), and 23.33 g 1-hydroxybenzotriazole (HOBt) in 1.2 L of dimethylformamide (DMF). The tips were then washed and deprotected using the usual methods. A proportion of the free amine groups on the tips were coupled to β-alanine by exposing the tips to a solution containing 26.19 g 9-fluorenylmethoxycarbonyl β-alanine, 17.07 g DCC, 25.27 g HOBt, and 3.56 g diisopropylethylamine in 1.37 L of DMF. After washing, the tips were assembled on their shafts and positioned in 12 x 8 matrices.

By contrast, 10000 of the rods or pins illustrated in Figure 1 were gamma-irradiated to graft polyacrylic acid to their entire surface using the earlier method

disclosed in International Patent Specification No. PCT/AU84/00039. Because of the greater bulk of the complete rod or pins, 11 L of the acrylic acid solution was required. After grafting, the rods or pins were assembled in 12 x 8 matrices and HMD coupled to the spherical tip of the rods. 1.2 L of solution as described above was prepared and dispensed in 150 µL quantities into microtitre trays. The rods were then inserted into this solution. This quantity was sufficient to couple HMD to 7230 rods. After washing and deprotection in the usual manner, β-alanine was coupled to the free amine groups using 735 mL of the solution described above. This quantity was sufficient to couple 4608 rods or pins.

The amount of free amine groups on the tips after coupling with HMD was 2230 nmole/tip whereas it was 1015 nmole/pin for rods or pins treated in the earlier way as disclosed in International Patent Specification PCT/AU84/00039. This difference in free amine level reflected the greater surface area of the "vaned" tips. This example also illustrates that use of the invention allows more active regions to be made at one time without the need to use more reagents and without the need to individually dispense reagents.

The hexapeptide, EYYLNH (using the single letter code to represent the amino acid residues in the hexapeptide), was synthesized on to 8 of each the rods or pins described above. This synthesis was carried out using the standard Fmoc chemistry for solid-phase synthesis of peptides. The peptides were then assayed in a standard enzyme-linked immunosorbent assay (ELISA) using an antibody which specifically reacted with the peptide. The active regions synthesized according to the invention gave a mean absorbance of 2.55 with a standard deviation of 0.104 (coefficient of variance = 4%). By contrast,

rods or pins treated in the earlier way as disclosed in International Patent Specification No. PCT/AU84/00039 gave a mean absorbance in the ELISA of 1.17 with a standard deviation of 0.116 (coefficient of variance = 10%).

This example illustrates the advantage of processing the active region of the rod or pin separately from the inactive region in substantially reducing rod-to-rod variation in testing.

It will be appreciated that many modifications and variations may be made to the specific embodiments described herein without departing from the broad principles of this invention as described herein, and all such modifications and variations are included within the ambit of this invention.

The claims defining the invention are as follows:

1. A solid surface for use in the synthesis of peptides or other polymeric compounds thereon, which surface comprises an active region on which said synthesis may take place and an inactive region on which said synthesis will not take place.

2. A solid surface of claim 1 wherein the surface is the surface of a rod having a portion providing the active region and remaining portion providing the inactive region.

3. A solid surface of claims 1 or 2 wherein the geometry of the active region is modified to provide a change in the surface area to volume ratio of the active region.

4. A solid surface of any one of claims 1 to 3 wherein the active region and the inactive region are manufactured as separate entities and then combined or assembled before use in the synthesis of peptides or other polymeric compounds.

5. A solid surface of claim 4 wherein the active region and the inactive region are manufactured by different processes or manufactured from different materials.

6. A solid surface of claims 4 or 5 wherein the active region is formed by coalescing or sintering together a large number of smaller solid particles.

7. A solid surface for use in the synthesis of peptides or other polymeric compounds thereon, which surface comprises an active region which is of a higher surface area/volume ratio than that of a cylinder of corresponding volume.

8. A solid surface according to Claim 7 wherein the active region comprises a porous material.

9. A solid surface according to Claim 7 or 8 wherein the active region comprises a plurality of relatively small particles joined together.

10. A solid surface substantially as herein described with reference to the examples and accompanying drawings.

FIGURE 1

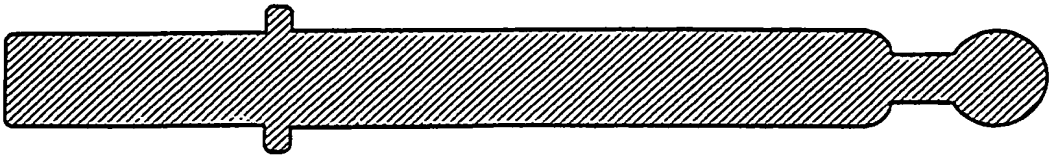


FIGURE 2

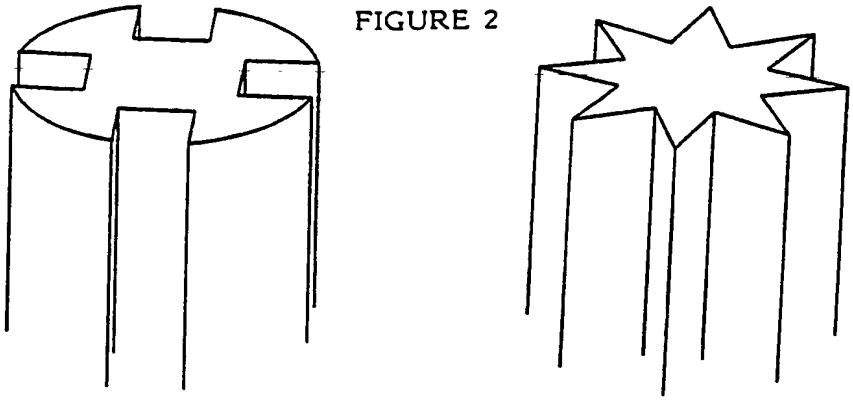
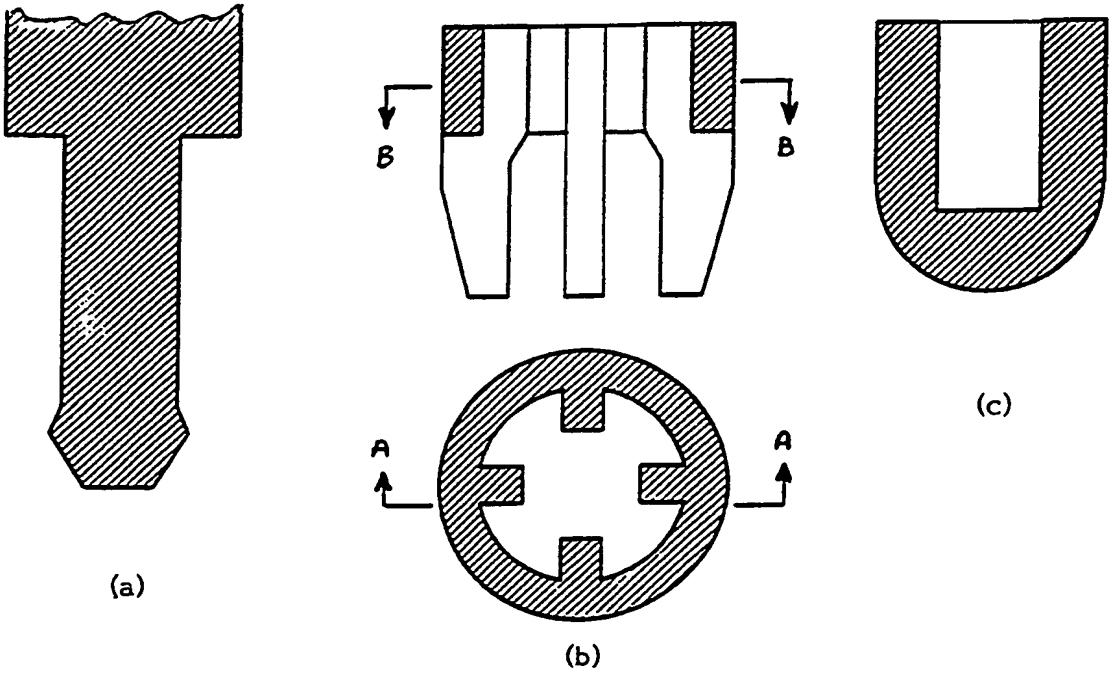


FIGURE 3



INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 90/00416

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[5] C07K 1/04, 3/04

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	Derwent, Keywords : Peptide, synthesis, rod or pin, solid phase, polyacrylic acid AU : IPC[5] C07K 1/04, 3/04

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

Chem. Abs., Keywords : Peptide, synthesis, rod or pin, solid phase, polyacrylic acid

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, [11] with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
X	AU,A, 76456/87 (STEEL, Samuel, L.) 22 September 1988 (22.09.88) see Drawing 1/1, claims, page 8, lines 5-8	(1)
X	US 4515920 (THE ROCKEFELLER UNIVERSITY / ERICKSON, B.W.) 7 May 1985 (07.05.85) see figures 1-6, claim 4	(1)
X	US 3557077 (BRUNFELDT, K. et al) 19 January 1971 (19.01.71) see abstract, claims	(1)
X	WO,A, 84/02526 (SOUTHERN ILLINOIS UNIVERSITY FOUNDATION) 5 July 1984 (05.07.84) see examples	(1)
X	AU,B, 12260/88 (BAYLOR COLLEGE OF MEDICINE) 27 July 1988 (27.07.88)	(1)

(continued)

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 November 1990 (20.11.90)	27 November 1990
International Searching Authority	Signature of Authorized Officer
Australian Patent Office	CEDRIC SCHAFFER

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X Y	DE,A, 3723004 (BOEHRINGER INGELHEIM KG) 26 January 1989 (26.01.89) see abstract, claims and drawings	(1-9) (10)
A Y	AU,A, 25429/84 (COMMONWEALTH SERUM LABORATORIES COMMISSION) 14 NOVEMBER 1985 (14.11.85) SEE CLAIMS 5-7	(1-9) (10)
P,A	AU,A, 38692/89 (BAYLOR COLLEGE OF MEDICINE) 25 January 1990 (25.01.90) see claims, drawings	(1-9)
P,X	AU,A, 42153/89 (MELDAL, M and BUCHARDT, O.) 22 March 1990 (22.03.90) see claims, drawings	(1)

V. [ ] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.[ ] Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2.[ ] Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.[ ] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

1.[ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.[ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.[ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 90/00416

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
AU 76456/87	IL 85698	US 4794150	WO 8807052
AU 38692/89	WO 9000626		
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WO 8402526	EP 131043	JP 60500535	
DE 3723004			
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US 4515020			

END OF ANNEX